Epithelial-to-mesenchymal transition of circulating tumor cells and CD133 expression on predicting prognosis of thyroid cancer patients

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Abstract. The present study aimed to explore the epithelial-to-mesenchymal transition of circulating tumor cells (CTCs) and CD133 expression in determining the prognosis of patients with thyroid cancer. It enumerated different CTC subtypes and analyzed CD133 gene expression in patients with thyroid cancer to evaluate the relationship between CTC number and thyroid cancer prognosis. In total 394 patients with thyroid cancer were enrolled. Among these, 270 cases had papillary thyroid cancer (PTC), 60 had follicular thyroid cancer (FTC), 30 had medullary thyroid cancer (MTC), 15 had poorly differentiated thyroid cancer, 19 had anaplastic thyroid cancer and 10 had non-malignant thyroid nodules based on their histopathological characteristics. CTC cell counts were determined by CanPatrol CTC capture technique before treatment. The present study also performed reverse transcription-quantitative PCR for CD133 gene expression and evaluated the relationship between CD133 expression and clinical pathology. A total of 330 cases of enrolled patients were classified as differentiated thyroid cancer, which included PTC and FTC. Their prognosis was excellent. The positivity rate of CTCs at diagnosis was 95.5%. The data of the present study showed that early recurrence and metastasis rates in PTC and FTC patients with >6 CTCs and positive mesenchymal circulating tumor cells (MCTCs) were significantly higher than those in patients with <6 CTCs and MCTCs. It was also found that those patients with >6 CTCs and MCTCs had shorter overall survival. In addition, CD133 levels in patients with thyroid cancer were strongly associated with the differentiation grades of thyroid cancers. The detection of >6 CTCs and positive MCTCs in patients with differentiated thyroid cancer was an excellent biomarker for predicting the prognosis of patients. CD133 expression was also identified as a good biomarker for thyroid cancer differentiation.

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Abbreviations: PTC, papillary thyroid cancer; FTC, follicular thyroid cancer; MTC, medullary thyroid cancer; PDTC, poorly differentiated thyroid cancer; ATC, anaplastic thyroid cancer; CTCs, circulating tumor cells; MCTCs, mesenchymal circulating tumor cells; NS, no significant difference; TNM, tumor node metastasis

Key words: CD133, circulating tumor cells, prognosis, progression free survival, thyroid cancer

Introduction

World wide, thyroid cancer is found in ~5% of women and 1% of men (1). Thyroid cancer is frequently identified during routine physical examinations using ultrasound imaging or fine needle aspiration biopsy (FNAB) (2). The final diagnosis of malignant thyroid nodules requires confirmation using histological examination of the excised thyroid tumor via surgery or FNAB (3). Therefore, a few methods, including specific gene detection using PCR, have been developed to preoperatively differentiate benign thyroid nodules (4). Thyroid cancers are divided into papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), medullary thyroid cancer (MTC), poorly differentiated thyroid cancer (PDTC) and anaplastic thyroid cancer based on their histopathological characteristics and original tissue resources (5). Normally, PTC and FTC together can be classified as differentiated thyroid cancer (DTC), which arises from follicular cells of the thyroid gland and has a more favorable prognosis than other type of thyroid cancer (6,7). By contrast, MTC, PDTC, and anaplastic thyroid cancers (8) arise from parafollicular cells and have a neuroendocrine origin. Treatments for patients with thyroid cancer include surgery, radioactive iodine-131 therapy, chemotherapy, hormone therapy and targeted therapy (9). Surgical resection and radioactive iodine-131 are no doubt effective therapies for non-metastatic thyroid cancers. However, radioactive iodine-131 therapy has little benefit in the treatments of MTC, PDTC and anaplastic thyroid cancers. Patients with unresectable nodules can be treated with external irradiation, which relieves pain in patients with bone metastases. The prognosis

of thyroid cancer is associated with its histopathological type and tumor-node-metastasis (TNM) staging. Normally, the overall prognoses of PTC and FTC are excellent. The overall 5-year survival rate is 85% for women and 74% for men at stages I-III, respectively (10,11). By contrast, patients with stage IV thyroid cancer and anaplastic thyroid cancer have a poor prognoses (11,12) because these patients with advanced thyroid cancer or low differentiated thyroid cancer do not respond to surgery or insensitive to radioactive iodine-131 treatment (13). Therefore, there is an urgent need to identify more sensitive and accurate biomarkers at an early stage for determining prognosis in patients with thyroid cancer.

Clinically, thyroglobulin can be used as a sensitive biomarker for monitoring DTC and most PDTC development. If a patient's thyroglobulin level is >0.3 ng/ml in serum after thyroidectomy, the change of relapse ismarkedly higher (14), but serial measurement in a shorttime limits its extensive application andanaplastic thyroid cancer, metastatic DTC and metastatic MTC may not produce thyroglobulin because of their poorly differentiated status. Recent studiesshowed that circulating tumor cells (CTCs) originate from the primary tumor and are released into the bloodstream, giving rise to tumor metastasis (15,16). Studies have demonstrated that CTC counts are a sensitive biomarker to predict tumor progression andhelp make treatment decisions (17-19). For example, Wang *et al* (20) show that higher CTC levels in patients with liver cancer are strongly correlated with early relapse. A review by Micalizzi *et al* (21) reported that epithelial cells from primary tumors can enter adjacent tissues via epithelial-mesenchymal transition (EMT) mechanism. CTCs are classified into epithelial, MCTC, and mixed types according to their surface markers (22). So far, CTC evaluation for thyroid cancer has little data to support its use, but available data revealed a slight correlation with CTC number (4,22,23). Therefore, new biomarkers need to be validated. Studies revealed that CD133, a glycoprotein encoded by the *PROM1* gene (24), is the most common marker of cancer stem cells (CSCs) from different carcinomas (25) and may be a good biomarker for predicting the prognosis of young patients with thyroid cancer (26). However, the detailed mechanisms remain to be elucidated.

The present study detected *CD133* gene expression and CTC levels in blood samples from patients with thyroid cancer and aimed to investigate the prognostic value of CTCs and CD133 expression inthyroid cancer.

Materials and methods

Patient samples. A total of 394 patients, including 270 cases papillary thyroid cancer (PTC), 60 follicular thyroid cancer (FTC), 30 of medullary thyroid cancer (MTC), 15 of poorly differentiated thyroid cancer (PDTC) and 19 of anaplastic thyroid cancer (8) as classified based on their histopathological characteristics, were involved in the present study between January 2018 and September 2020. Another 10 patients without thyroid tumors were used as the negative controls. Their age ranged between 9-82 years. Males made up 149 of the cases and females 245 cases. All samples were collected from patients' peripheral blood at diagnosis and before treatment. All patients were followed up at every five months. Patients with possible recurrence were followed up every two months. Overall survival (OS) was calculated as the time from initial diagnosis to patient mortality. The study protocol was approved by the ethics committee of the Affiliated Cancer Hospital of Zhengzhou University (approval no. 2022-KY-0009-001). Written informed consent was obtained from all the participating patients prior to sample collection.

Characterization of CTCs using CanPatrol and tricolor RNA-ISH methods. Characterization strategies using CTC in patients with thyroid cancer were followed as described in the literature (27). A total of 5 ml of peripheral blood was collected from the patients at diagnosis and the control participants. Each sample was then spun for 5 min at 300 x g at room temperature (RT) for 4 h after collection. The upper plasma phase was discarded and CTCs were isolated using CanPatrol CTC enrichment technique (SurExam Bio-Tech Co., Ltd.). For CanPatrol CTC enrichment procedure, the above cells were mixed with 15 ml erythrocyte lysis buffer (cat. no. 00-4333-57; Thermo Fisher Scientific, Inc.) and incubated for 30 min at RT. Then, it was centrifuged for 5 min at 350 x g at RT and the supernatants discarded. Cells were fixed for 15 min with cytofix/cytoperm fix solution (cat. no. 554722, BD Biosciences) at 4˚C and were transferred to a filter tube with an 8 μm pore size filter membrane for filtering with a vacuum pump. Cells were further fixed at RT for 1 h by 4% paraformaldehyde (PFA).

Following CTC enrichment, Alexa Fluor 594 labeled epithelial makers (EpCAM, CK8/18/19), Alexa Fluor 488 conjugated mesenchymal markers (vimentin and twist) and nuclear makers (4',6-diamidino-2-phenylindole, DAPI) were used to identify CTCs along with a tri-color RNA *in situ* hybridization technique (28). Briefly, the enriched CTCs were treated with 0.1% mg/ml proteinase K to increase cell membrane permeability. Then, capture probes were mixed for hybridization at 40˚C for 2 h and unbound probes were washed with 0.1X SSC solution. To amplify the probe signal, the pre-amplification and the amplification solution were added to the hybridization solution. CTCs were classified into epithelial, mesenchymal and mixed types according to the combination of their surface markers with DAPI (Fig. 1). Following capture probes hybridization and signal amplification, cells were stained with DAPI and counted under a fluorescence microscope (Olympus BX53; Olympus Corporation). Epithelial CTCs were identified with Alexa Fluor 594 labeled epithelial makers (EpCAM/CK8/18/19) and showed red color dots under the microscope (Fig. 1A). MCTCs were counted with Alexa Fluor 488 conjugated mesenchymal markers (vimentin and twist) probes and revealed green color dots under the microscope (Fig. 1B). If there were red and green mixed dots in cells, these cells were counted as mixed CTCs (Fig. 1D). Cell nuclear images were marked with DAPI staining (Fig. 1C).

CTCs counting criteria. Following the above CTCs markers, different CTC subset criteria were set up. The red dots, green dots and mixed dots were counted at x100 magnification using different wavelengths with an automated imaging fluorescent microscope (Carl Zeiss AG). Positive and negative cells were counted for the three types of CTCs in seven fields of

Table I. Basic demographic and clinicopathological characteristics.

Clinicopathological characteristic	No of patients	Age	Sex, female (%)	Tumor size range (cm)
Control	10	43.8 (19-75)	67	2.0 (0.2-1.0)
MAL	394	44.6 (9-82)	66.5	2.0 (1.2-2.8)
MAL-papillary	270	48.6 (9-75)	74	2.3 (1.0-4.8)
MAL-follicular	60	46.5 (15-81)	68	2.3 (1.1-5.5)
MAL-medually	30	44.5 (13-75)	75	2.1 (1.0-3.0)
MAL-poorly differentiated	15	40.7 (15-68)	60.3	2.0 (1.3-2.8)
MAL-anaplastic	19	41.5 (15-65)	67	2.5 (1.8-3.5)
I	154	42.7 (9-82)	64.6	
II	50	46.7 (13-75)	68	
III	54	47.1 (15-76)	63.7	
IV	136	44.9 (15-81)	65.7	

MAL, malignant; I, II, III and IV, tumor-node-metastasis stages.

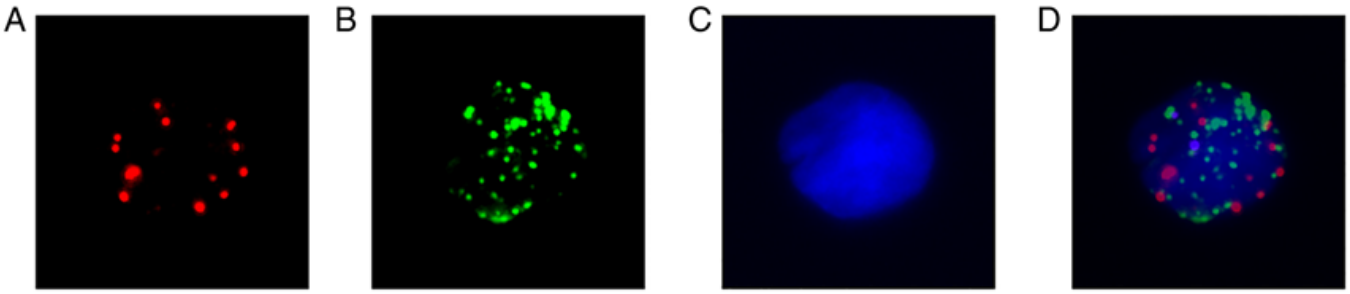

Figure 1. Images of CTCs. (A) epithelial CTC image, (B) mesenchymal CTC image, (C) cellular nucleus with DAPI staining and (D) merged image. Epithelial CTCs were stained with only Alexa Fluor 594 labeled epithelial markers EpCAM and CK8/18/19. Mesenchymal CTCs were stained with Alexa Fluor 488-labeled vimentin and twist. Images were capturedat 100x magnification with a fluorescence microscope. CTCs, circulating tumor cells; DAPI,4', 6-diamidino-2-phenylindole.

view (DAPI positive cells) manually under the fluorescent microscope.

CD133 expression by reverse transcription-quantitative (RT-q) PCR. Whole peripheral blood (5 ml) was obtained from the patients with thyroid cancer and negative control. Mononuclear cells (MNC) were isolated via lymphocyte Ficoll separation solution at 300 x g for 90 min at RT and cell density adjusted to $1x10^6$/ml for total 2 ml. Next, 1 ml TRIzol (Thermo Fisher Scientific, Inc.) was added. Total RNA was extracted using RNeasy kit (cat. no. 74004; QiagenGmbH) and cDNA synthesis performed using commercial reagents (cat. no. K1621; Thermo Fisher Scientific, Inc.) following manufacture's protocol. The CD133 gene PCR reagents were purchased from Thermo Fisher Scientific and were performed quantitative PCR using SYBR Green Master Mix (Thermo Fisher Scientific, Inc.). For human CD 133 and GAPDH primer sequences were following: CD133 forward primer, 5'-AGTCGGAAA CTGGCAGATAGC-3'; reverse primer: 5'-GGTAGTGTT GTACTGGGCCAAT-3'; GAPDH forward primer, 5'-GGA GCGAGATCCCTCCAAAAT-3'; GAPDH reverse primer: 5'-GGCTGTTGTCATACTTCTCATGG-3'. Human CD133 and GAPDH gene ID #:NM_001145847 and NM_001256799 were entered into pga.mgh.harvard.edu/cgi-bin/primerbank. Thermocycling conditions were: Denaturing 95˚C for 5 min; 95˚C for 30 sec, 56˚C for 30 sec, 72˚C for 1.5 min, 35 cycles; 72˚C for 5 min; 4˚C for 1 h. CD133 expression was calculated by the $2^{-\Delta\Delta Cq}$ method and normalized to GAPDH (29). The results were from three independent experiments.

Statistical analysis. The association between CTC levels and clinicopathological profiles were evaluated by the χ^2 test. CTCs levels were compared by Dunn's test following Kruskal-Wallis test. OS was calculated as the time from initial diagnosis to death at cut-off time using the Kaplan-Meier method and log-rank test. CD133 expression in different thyroid cancer subsets was performed using χ^2 test. All results were analyzed using GraphPad Prism 8 software (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinical characteristics. The present study enrolled 394 thyroid cancer patients with T1-4TNM stages and 10 negative controls. Clinicopathological features of the patients are presented in Table I. The clinical parameters included age, sex, histology, differentiation grade and TNM

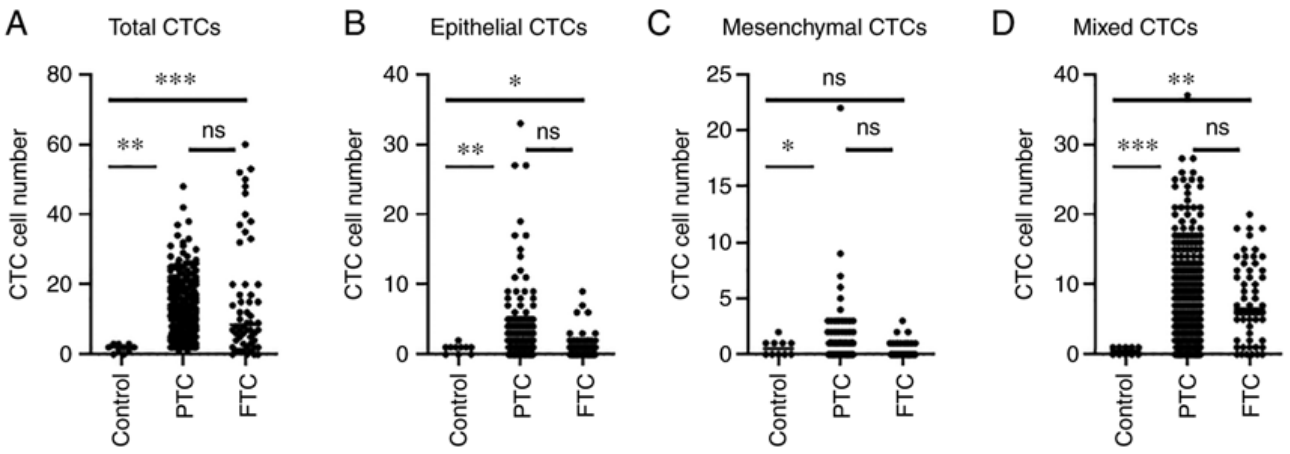

Figure 2. Comparison of CTCs and subtypes CTC in PTC, FTC and control patients. (A) total CTC count comparison, (B) Epithelial CTC count comparison, (C) MCTC count comparison and (D) mixed CTC count comparison. **P<0.01; ***P<0.001; *P<0.05. CTCs, circulating tumor cells; PTC, papillary thyroid cancer; FTC, follicular thyroid cancer; CTCs, circulating tumor cells; MCTC, mesenchymal circulating tumor cell; NS, no significant difference.

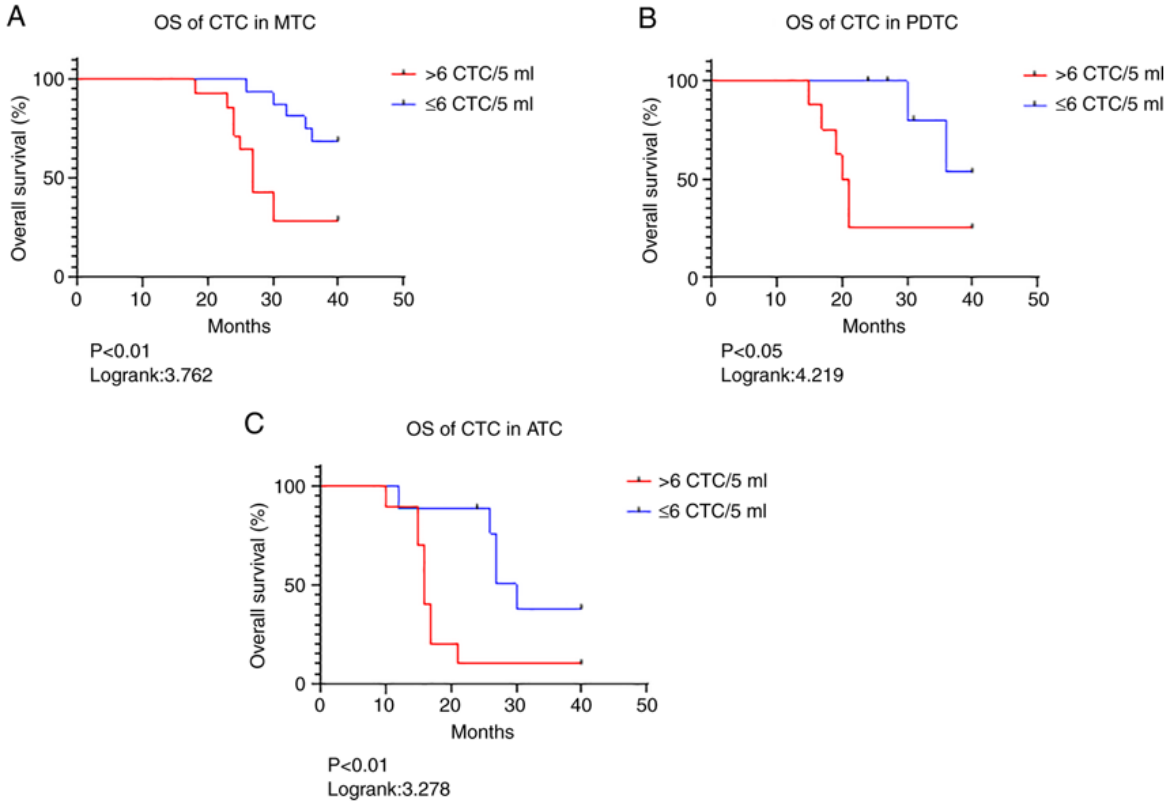

Figure 3. OS of patients with CTCs and MCTCs by Kaplan-Meier curves at diagnosis. (A) OS in >6 CTCs vs. ≤6 CTCs in MTC patients. (B) OS in >6 CTCs vs. ≤6 CTCs in PDTC patients. (C) OS comparison in >6 CTCs vs. ≤6 CTCs in ATC patients. OS, overall survival; CTCs, circulating tumor cells; MCTC, mesenchymal circulating tumor cells; MTC, medullary thyroid cancer; PDTC, poorly differentiated thyroid cancer; ATC, anaplastic thyroid cancer.

stages. Among the patients, most patients were diagnosed with papillary thyroid cancer (PTC; 69.8%) and follicular thyroid cancer (FTC; 14.2%). In addition, patients with medullary thyroid cancer (MTC; 7.6%), poorly differentiated thyroid cancer (PDTC; 3.7%) and anaplastic thyroid cancer (4.7%) were also included the present study. Among the different subtypes, the number of female patients (265; 67.3%) was almost double than that of the male patients (129; 32.7%). There were no significant differences in patient age. Tumor sizes ranged from 0.123-33.2 cm^3.

Identification of CTC subtypes in patients with thyroid cancer. Peripheral blood (5 ml) from 394 patients with thyroid cancer and 10 healthy controls were used to identify CTC subtypes using CanPatrol and tricolor RNA-ISH method. This method has some advantages over the other techniques for CTC detection: i) It can measure the frequency of relatively fewer cells; ii) it allows for detection of multiple genes in a single CTC; and iii) it can assess the EMT of CTCs and predict the prognosis of cancer (30). CTCs were classified into epithelial, mesenchymal and mixed subtypes based on their surface

Table II. Comparison of OS on MTC, PDTC and ATC patients.

Variables	HR	95% CI	P-value
CTC in MTC >6 vs. ≤6/5 ml	3.762	1.299-10.89	<0.01
CTC in PDTC >6 vs. ≤6/5 ml	4.219	1.034-13.2	<0.05
CTC in ATC >6 vs. ≤6/5 ml	3.278	1.077-9.8	<0.01

OS, overall survival; MTC, medullary thyroid cancer; PDTC, poorly differentiated follicular thyroid cancer; ATC, anaplastic thyroid cancer; HR, hazard ratio; CI, confidence interval; CTC, circulating tumor cell.

markers with different immunofluorescent dye staining (Fig. 1). The data revealed that most patients had only one type of CTC. Few epithelial CTCs were detected in the benign control group. CTCs were found 376 out of 394 thyroid cancer patients (95.4%). All patients with MTC, PDTC and ATC had detectable levels of CTCs.

Characteristics of CTCs in patients with thyroid cancer. To assess the clinical significance of CTC number in patients with different types of thyroid cancer, the total CTCs and CTC subtypes of major differentiated thyroid cancers PTC and FTC were compared because MTC, PDTC and ATC are undifferentiated thyroid cancer and have a short OS. The results are shown in Fig. 2. Total CTCs and mixed CTCs in either PTC or FTC are dramatically higher than those in control (Fig. 2A, P<0.01). However, there were no significant differences between the PTC and FTC groups (Fig. 2A). In epithelial CTCs (Fig. 2B), dramatically higher than the control (P<0.01) and FTC also was markedly more than the control (P<0.05). By contrast, MCTC (Fig. 2C), MCTC count in PTC patients were significantly higher than those in the controls, but there was no obvious difference between FTC patients and controls. Similar to total CTCs, mixed CTCs (Fig. 2D) in either the PTC or FTC group were significantly higher than those in the control group.

Prognostic significance of CTC counts and subtypes. The prognosis of patients with DTC is generally excellent. Therefore, the clinical significance of CTC subtypes in poorly differentiated thyroid cancers, such as MTC, PATC and ATC was further investigated and followed up to 60 months for patient prognosis. The results are presented in Fig. 3 and Table II. The OS in the patients with MTC (Fig. 3A); PDTC (Fig. 3B) and ATC (Fig. 3C) was investigated. In MTC patients, OS when CTCs >6 was significantly shorter (P<0.01) compared with patients with CTCs ≤6 according to the Kaplan-Meier's survival curve analysis. The hazard ratio (HR) (31) and 95% confidence interval (CI) were 3.762 and 1.299 to 10.89, respectively. In the patients with PDTC, the HRs and 95% CIs were 4.219 and 1.034 to 17.2 (P<0.05). By contrast, HR and 95% CI were 3.278 and 1.077 to 9.8 (P<0.01) in ATC patients (Table II). These results indicated that high CTC numbers in patients with PDTC were a powerful biomarker for predicting the prognosis of thyroid cancer.

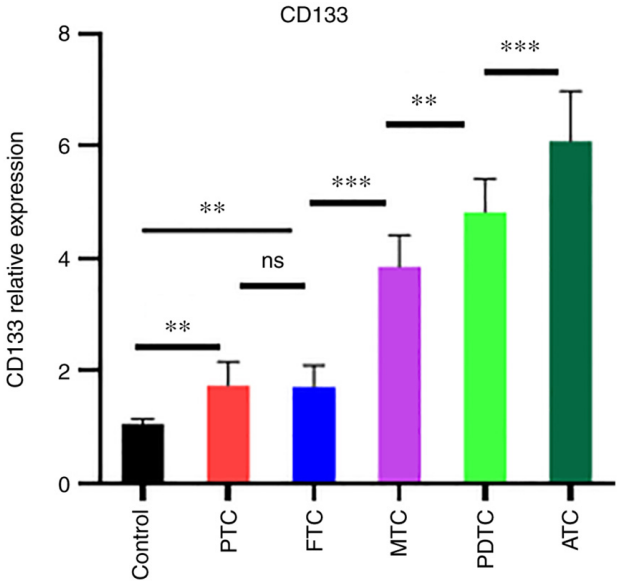

Figure 4. CD133 expressions in thyroid cancer subtype. Data shows CD133 relative expression by reverse transcription-quantitative PCR. ***P<0.001; **P<0.01. ns, no significant difference; PTC, papillary thyroid cancer; FTC, follicular thyroid cancer; MTC, medullary thyroid cancer; PDTC, poorly differentiated thyroid cancer; ATC, anaplastic thyroid cancer.

CD133 expression is significant relevant to thyroid cancer differentiation. To evaluate the relationship between CD133 expression and thyroid cancer differentiation, CD133 gene expression was measured using qPCR in the thyroid cancer subtype. The results are shown in Fig. 4. CD133 expression was higher in poorly differentiated cells. ATC and PDTC showed robust expression compared to the control (P<0.001). By contrast, PTC or FTC showed high expression compared to control (P<0.01). Notably, CD133 expression was also significantly higher in ATC than in PDTC (P<0.001). CD133 expression in PDTC was higher than that in PTC and FTC (P<0.01). These results revealed that CD133 expression is strongly associated with the degree of thyroid cancer differentiation.

Discussion

Studies show that CTCs are strongly associated with cancer development (21,32). A number of clinical studies have revealed that the CTC count of the peripheral blood in patients with advanced stages of cancer is an important guideline for predicting patient prognosis (17-19). A few reports have indicated that CTCs of patients with thyroid cancer are involved in disease progression (22,23,33). However, data investigating CTCs in patients with thyroid cancer at early stages are limited. The present study showed that the total CTCs and their subtypes had a significant clinical association with the prediction of thyroid cancer prognosis.

CTCs in the blood stream can often be identified as epithelial, mesenchymal, or both mixed types according to their surface markers with different immunofluorescence stains (34,35). Studies indicate that EMT marker expression in CTCs in a number of types of cancer, such as gastric cancer, colorectal cancer, non-small cell lung cancer, breast cancer and prostate cancer are relevant to invasion and metastasis (36-39).

Previous studies show that CTCs in thyroid cancer can be detected with immunochemical staining of EpCAM epithelial marker (23) or based on cell size (40) and antibody capture (41). However, these methods have low sensitivity and are less reliable than RNA-ISH. Antibody capture needs ≤27.5 ml peripheral blood. By contrast, the RNA-ISH has very high sensitivity and specificity in only 5 ml peripheral blood. It was found that if the total CTC and MCTC counts were high at diagnosis, patients were more likely to have rapid tumor progression. The present study also showed that if patient blood samples had <6 MCTCs, OS of patients with thyroid cancer were significantly longer than that of patients with >6 CTCs. Similarly, patients with an increased MCTC percentage following surgery relapse earlier in hepatocellular cancers (42). Finding from past studies support that CTC monitoring identifies not only the nature of the tumor, but also provides the underlying biology of tumor recurrence and metastasis (43-45). For example, the presence of CTCs is closely associated with metastasis of small cell lung cancer (46). de Sousa e Melo *et al* (47) found that a high number of CTCs indicates relapse in patients with colorectal cancer. Therefore, the detection of CTCs, EMT CTCs and changes in patients with thyroid cancer may provide another predictor of recurrence compared with conventional clinical parameters.

In addition to the clinical significance of CTCs, a number of studies have also explored other biomarkers for the prognosis of thyroid cancer (48-50). Among these biomarkers, CD133 is an interesting gene because it is involved in a number of types of cancers (51-53). Ge *et al* (54) reveal that targeting CD133 may greatly improve the prognosis of ATC. This result shows that high CD133 expression promotes thyroid cancer proliferation. Indeed, the present study indicated that higher CD133 levels were strongly associated with poorly differentiated thyroid cancer. These data confirm that CD133 is also a good biomarker for the diagnosis and therapy of thyroid cancer.

The present study indicated that CTCs in peripheral blood were strongly associated with OS in patients with thyroid cancer. High levels of CTCs or MCTCs were significantly correlated with early recurrence or metastasis. CD133 is a new biomarker for the diagnosis and therapy of thyroid cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YD conceived and designed this study; DL and NL performed the experiments and analyzed the data. DL, NL and YD drafted the manuscript. DL and YD confirm the authenticity of all the raw data. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

All human thyroid cancer blood samples were approved by the ethical committees of the Affiliated Cancer Hospital of Zhengzhou University (approval no. 2022-KY-0009-001). A written informed consent was obtained before study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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